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Stimulation by Ionophores of Tyrosinase Activity of Mouse Melanoma Cells in Culture

HISAAKI SAEKI, PH.D. AND ATSUSHI OIKAWA, PH.D.

Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan

The hypothesis that the melanosome is an acidic vesicle in which the tyrosinase action is suppressed under the ordinary culture conditions was examined with a variety of ionophores added in cultures of mouse melanoma cell line B16-C_2M.

In the presence of monensin or nigericin, which exchange H^+ for Na^+ or K^+, respectively, through biomembrane, the tyrosinase activity of cells in culture was more than 10 times that in the control culture. This stimulation was observed without delay after addition of the chemicals and was not inhibited by cycloheximide. The enzyme activity of sonicated cell-free extracts, in which melanosomes were disrupted, was not stimulated by these ionophores.

The tyrosinase activity was stimulated to a lesser extent by a proton ionophore, *p*-trifluoromethoxyphenylhydrazone (FCCP). The activity was also stimulated by kryptofix 221, valinomycin (Na^+ and K^+ carrier, respectively), and tetraethylammonium ions (permeant cations) but only in the presence of a limited concentration of FCCP. N-Ethylmaleimide and N,N′-dicyclohexylcarbodiimide, inhibitors of lysosomal proton pump, stimulated tyrosinase activity of cells in the presence of FCCP.

These facts are consistent with the hypothesis described above.

The melanocyte is a specialized cell for melanin synthesis catalyzed by tyrosinase (EC 1.14.18.1), which is confined in the melanosome, an intracellular vesicle unique to the melanocyte [1]. In a previous paper [2], we proposed a hypothesis based on the cellular responses to lysosomotropic agents that the melanosome is an acidic vesicle, in which the pH is suboptimal for tyrosinase under the usual culture conditions of a near-neutral pH, and lysosomotropic agents provide a better condition for the enzyme action due to degeneration of the transmembrane pH gradient of melanosome.

If the melanosome is really an acidic vesicle, treatments that collapse the transmembrane pH gradient should increase the tyrosinase activity in situ. In the present study we examined the effect of various types of ionophores and compounds related to ion transport through biomembrane [3] on tyrosinase activity in melanoma cell cultures. Results were consistent with our expectations.

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Reprint requests to: Hisaaki Saeki, Ph.D., Research Institute for Tuberculosis and Cancer, Tohoku University, 4-1 Seiryomachi, Sendai 980, Japan.

Abbreviations:
DCCD: N,N′-dicyclohexylcarbodiimide
FCCP: *p*-trifluoromethoxyphenylhydrazone
MEM: minimum essential medium
NEM: N-ethylmaleimide
TyC: tyrosinase activity of living cells in culture
TyH: tyrosinase activity of sonicated cell-free extracts

MATERIALS AND METHODS

Chemicals

L-[3,5-^{3}H]Tyrosine (45 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, England). The mixture of amino acids and vitamins for Eagle's minimum essential medium (MEM) was purchased from Nissui Seiyaku Co. (Tokyo); calf serum from Flow Laboratories (North Ryde, Australia); cycloheximide from Upjohn Co. (Kalamazoo, Michigan).

Monensin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and valinomycin were purchased from Sigma Chemical Co. (St. Louis, Missouri); nigericin was from Calbiochem-Behring Co. (La Jolla, California); 4,7,13,16,21-pentaoxa-1,10-diazabicyclo[8.8.5]tricosane(kryptofix 221) from E. Merck Japan Ltd. (Tokyo); N-ethylmaleimide (NEM) from Wako Pure Chemical Industries (Osaka); and N,N′-dicyclohexylcarbodiimide (DCCD) from Tokyo Kasei Kogyo Co. (Tokyo). Soluene 350 was a product of Packard Instrument Co. (Downers Grove, Illinois).

Cells and Cell Cultures

B16-C_2M mouse melanoma cells [4] were cultured in Eagle's MEM containing 2 mM glucose and supplemented with 10% calf serum as described previously [2]. Cells were inoculated into plastic flat-bottomed culture tubes (5 cm^2) at 2–3 × 10^5 cells/culture for experimental cultures. Medium (1 ml, pH 7.4) was renewed daily. Cultures 48 h after inoculation (5–8 × 10^5 cells/culture) were used for experiments.

Assay Methods

Tyrosinase activities of living cells in culture (TyC) and of sonicated cell-free extracts (TyH) were assayed by measuring the production of radioactive water from L-[3,5-^{3}H]tyrosine as described previously [5,6].

Culture period for TyC assay was 3 h, if not otherwise stated. TyC was determined for 4 identical cultures and the mean and standard error were presented as percentage of the activity assayed in the presence of 10 μM monensin, which gave the highest stimulation among ionophores examined. Because TyC stimulated by 10 μM monensin differed in separate experiments, ranging from 1.2–9.3 nmol/culture/h, depending on the basal activities and cell population densities at the start, cultures containing monensin were included in all experiments as a reference.

TyH assay for pH-activity profile was carried out in 80 mM sodium phosphate buffer of various pH's.

The amount of melanin was assayed by dissolving a washed cell pellet directly in Soluene 350 and measuring the absorbance at 400 nm as described previously [7,8]. Protein was assayed by the method of Lowry et al [9] with bovine serum albumin as a standard.

RESULTS

When 10 μM monensin was added to near-confluent cultures of B16-C_2M mouse melanoma cells, the rate of tyrosine hydroxylation in culture increased to a level more than 10 times that in control cultures. As shown in Fig 1*A*, this increase took place without delay and the hydroxylation proceeded in a nearly linear fashion for at least 3 h. This stimulation was not inhibited by cycloheximide. These facts indicate that the increase in tyrosinase activity of culture (TyC) is not due to the de novo synthesis of tyrosinase but is due to an activation of the enzyme. Thus, assays of tyrosinase activity presented hereafter were carried out in a short period (3 h) without the addition of cycloheximide.

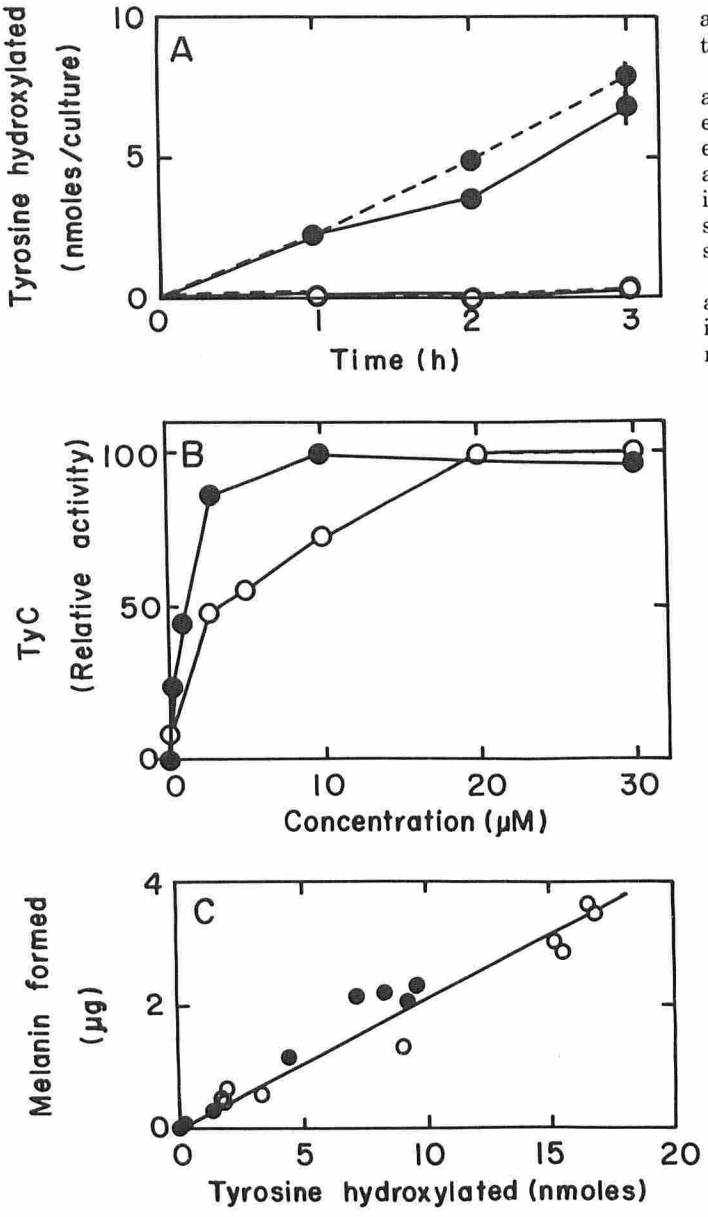

FIG 1. *A*, Stimulation by monensin of hydroxylation of tyrosine in culture of B16-C_2M mouse melanoma cells. Cells were incubated with (●) and without (○) 10 μM monensin in the presence (*broken line*) and absence (*solid line*) of 1 μg/ml of cycloheximide for indicated times. Each point represents the mean and standard error of assays for 4 cultures. *B*, Dose-dependent activation of tyrosinase by monensin and nigericin. Tyrosinase activity was expressed as a percentage of the activity stimulated by 10 μM monensin. Each point represents the mean for 4 cultures. Standard errors at all points were smaller than the size of circles. ●, monensin; ○, nigericin. *C*, Proportional relationship between hydroxylation of tyrosine and production of melanin in culture activated by ionophores. Results from 2 separate experiments with monensin (●) and FCCP (○) were plotted.

The activation of TyC by monensin was concentration-dependent, and detectable at a concentration as low as 0.3 μM (Fig 1*B*). The least concentration of monensin to give the maximum activation was about 10 μM. Nigericin activated tyrosinase to a level similar to that monensin did, but at a higher concentration. Under these activating conditions, the amount of melanin produced was proportional to the amount of tyrosine hydroxylated (Fig 1*C*).

This stimulation was examined with cells cultured in media of a range of pH's in the presence of 10 μM monensin. The pH activity profile more resembled that of cell-free extracts, than that of cultured cells without monensin (Fig 2).

FCCP stimulated TyC to a lesser extent than did monensin as shown in Table I. Kryptofix 221, valinomycin, and tetraethylammonium ions showed no stimulation, but in the presence of a suboptimal concentration of FCCP these chemicals activated TyC. NEM and DCCD stimulated tyrosinase activity in the presence of FCCP. In the case of DCCD, however, stimulation was also observed in the absence of FCCP. The stimulation in all these cases was dose-dependent (Table I).

Tyrosinase activity of sonicated cell-free extracts was not affected by any of these chemicals at the concentrations used in experiments with cultured cells, except by FCCP, which reduced the activity to 90% of the control.

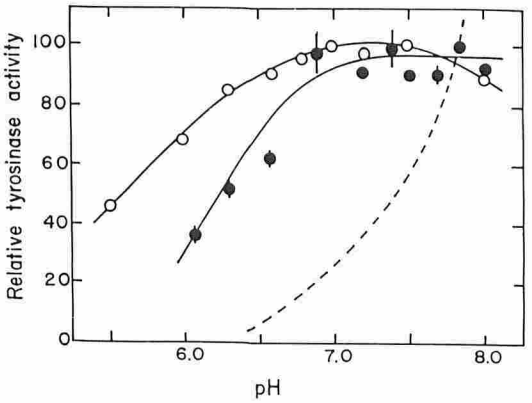

FIG 2. pH-tyrosinase activity profiles of cells in culture and of cell-free extracts. TyC's of nearly confluent cells were assayed by incubating them for 3 h in culture media of various pH's in the presence of 10 μM monensin (●). The pH values indicated are the ones at the end of incubation, the original pH's of the points on the right were slightly higher than those indicated. TyH's at indicated pH's were obtained with sonicated extracts of cells collected without any treatment (○). Activities are expressed as percentages of the highest one for respective curves. Each symbol and bar is the mean ± SE of 4 determinations. The corresponding TyC curve in the absence of monensin (*broken line*) was cited from a previous paper [2] after recalculation to compare with the present curves.

TABLE I. *Stimulation of TyC by ionophores, a permeable cationic compound, and inhibitors of lysosomal proton pump*

Chemicals	Concentration	TyC (% of monensin-stimulated TyC)	
FCCP (H^+-ionophore)	0 μM	6.9 ± 0.1	
	40	28.8 ± 2.1	
	80	60.0 ± 0.5	
		No FCCP	40 μM FCCP
Kryptofix 221 (Na^+-ionophore)	0 μM	11.2 ± 1.1	35.7 ± 1.0
	10	8.7 ± 0.8	46.1 ± 2.2
	20	9.3 ± 1.3	60.5 ± 1.3
	40	10.0 ± 0.9	63.0 ± 1.7
Valinomycin (K^+-ionophore)	0 μM	7.0 ± 1.2	14.1 ± 1.2
	20	−1.5 ± 0.6	41.6 ± 0.5
	40	−3.5 ± 1.9	49.5 ± 4.0
Tetraethyl-ammonium-Cl (permeant cation)	0 mM	−0.2 ± 0.5	23.7 ± 1.0
	30	−0.3 ± 0.9	42.1 ± 0.8
	50	2.3 ± 1.1	53.8 ± 3.3
N-Ethylmaleimide (H^+ pump inhibitor)	0 μM	2.5 ± 1.3	14.1 ± 1.5
	5	2.2 ± 1.3	16.4 ± 0.8
	10	−0.7 ± 0.4	22.2 ± 0.4
DCCD (H^+ pump inhibitor)	0 μM	3.7 ± 0.9	7.8 ± 0.4
	50	6.1 ± 1.1	31.2 ± 2.0
	100	21.3 ± 2.2	46.9 ± 2.4

The means ± SE of TyC's for 12 monensin-stimulated and 12 control runs, each of which consists of 4 cultures, were 4.9 ± 0.6 and 0.18 ± 0.06 nmol/10^6 cells/h, respectively.

DISCUSSION

In this study we examined the effect of 5 functionally distinct ionophores on tyrosinase activity of melanoma cells in culture (TyC). Among these ionophores the most effective ones were monensin and nigericin, which facilitate the transmembrane flow of both protons and univalent metal ions in an exchanging fashion. FCCP, a proton carrier, was less effective. Univalent metal ionophores, kryptofix 221 and valinomycin, gave a positive effect only in the presence of FCCP.

The inferior effect of FCCP to proton/metal ion exchange ionophores would be due to an electric potential produced by the outward flow of protons, which obstructs the further proton flow and possibly activates the proton pump as seen in chromaffin granules [10], resulting in insufficient neutralization of intramelanosomal pH, while monensin and nigericin exchange proton for the metal ion keeping electroneutrality, resulting in no obstruction of neutralization of the intravesicular pH. The metal ionophores do not transport protons, but would be effective in eliminating the electric potential produced by the FCCP-driven outward flow of protons. The ability of tetraethylammonium ions would be explained by analogy to the metal ionophores.

The resemblance of the pH-activity profile of TyC determined with monensin-treated cultures to that of TyH with cell extracts indicates that the ionophore has, as expected, an effect equivalent to the disruption of biomembrane barrier by sonication.

NEM and DCCD, inhibitors of ATP-driven proton pump, would not have a direct effect on raising internal pH, but would inhibit the compensation by the pump of FCCP-directed proton release, resulting in the stimulation of tyrosinase. The reason for stimulation by DCCD without FCCP is unknown.

Another possibility for the stimulation to be kept in mind is that the proton-releasing ionophores increase concentrations of tyrosine and/or oxygen, the substrates of tyrosinase, in melanosomes by unknown mechanisms, resulting in the stimulated tyrosinase action, although it is not very likely because of the very high degree of stimulation observed.

In addition to a variety of acidic vesicles equipped with proton pump [10–14], Golgi vesicles and rough and smooth endoplasmic reticula have been found to belong to the same category of organelles in this respect [15,16]. Because the melanosome is derived from these organelles [17–19], the proton pump could possibly be provided by membrane systems of these organelles.

In melanocytes melanosomes are sometimes found as the compound melanosome, which consists of several melanosomes and surrounding vesicular membrane and looks like a phagosome. This outer membrane might be provided by digestive vesicles such as lysosomes, and the mechanism postulated above could exist on this membrane instead of the melanosomal membrane. This possibility was ruled out by an electron microscopic study (to be published), in which we found scarcely any compound melanosomes in the melanoma cells used in the present study. It was also found that in monensin-treated cells there were more melanosomes of stages III and IV and fewer stage II melanosomes, defined by Toda and Fitzpatrick [17], when compared to control cells, keeping the total number of melanosomes of stages II, III, and IV nearly unchanged. The latter findings indicate that monensin stimulates the synthesis of melanin in melanosomes.

The above discussion on the acidic nature of intramelanosomal space and differential responses to different ionophores is based on an assumption that the cytoplasmic pH is similar to the medium pH under the near-neutral environment as it usually is in various types of cells. Ionophores are cytotoxic due to destruction of the physiologic ionic balance of cells, but biochemical understanding of what underlies the stimulation of TyC by lysosomotropic agents and ionophores should be useful for developing therapeutics of melanogenic disorders. The proposition that intramelanosomal pH is kept acidic by a proton pump still remains to be proved by experiments with cell-free and intact melanosome preparations.

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